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March 10, 1993

Mr. Alan Morrison, Procurement Assistant
Department of the Navy
Office of Naval Research
Stanford University
McCullough Building, Room 202
Stanford, CA 94305-4055



RE: N00014-91-J-1815

Final Technical Report for this grant.

The goal of this grant was to isolate and characterize the antifouling substances from seaweeds provided by the group at Hopkins Marine Station, Pacific Grove, Calif. Overall, the objectives were accomplished. Zosteric acid was isolated and identified as the key active principle of the alga. A synthesis was developed and the group at Hopkins were trained to carry-out this synthesis work.

One staff researcher, visiting professor James Todd was supported by this grant. Two manuscripts were produced from Dr. Todd's experimental work. Additional details about these publications is as follows: (1) "The isolation of a monomeric carboxylic acid of swinholide A from the Indo-Pacific sponge, *Theonella swinhoei*", James S. Todd, K. A. Alvi, and Phillip Crews, *Tetrahedron Letters*, Vol. *33*, No. 4, pp. 441-442, **1992**. (2) "The antifouling activity of natural and synthetic phenolic acid sulfate esters." James S. Todd, Richard C. Zimmerman, Phillip Crews, and Randall S. Alberte, *Phytochemistry*, in press (1993).

A patent application has also been filed which discloses the major findings about Zosteric acid. Development work is still in progress on this very important antifouling lead. Additional details concerning the patent are: Title: "Phenolic acid sulfate esters for prevention of marine biofouling." Inventors: Richard C. Zimmerman, Randall S. Alberte, James S. Todd, Phillip Crews. UC case No. 92-002. The agency filing for the patent is ARCH Development Corp., Argonne, Illinois, Project Number 61571-00.

Sincerely,

Phil Crews
Professor of Chemistry

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CONTRACTS AND GRANTS OFFICE
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June 2, 1993

Scientific Officer Code: 1213
Stephen L. Snyder
Office of Naval Research
800 North Quincy St.
Arlington, VA 22217-5000

re: ONR Grant N00014-91-J-1815
Dr. Phillip Crews, Principal Investigator

Dear Dr. Snyder:

Enclosed you will find three (3) copies of the final technical report for the above-referenced grant.

If you need any additional information, please contact me at 408/459-2779.

Sincerely,

Anne Gavin
Associate Research Administrator

Enclosure

1 Copy to: Administrative Contracting Officer
Office of Naval Research
Resident Representative N63375
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Stanford, CA 94305-4055

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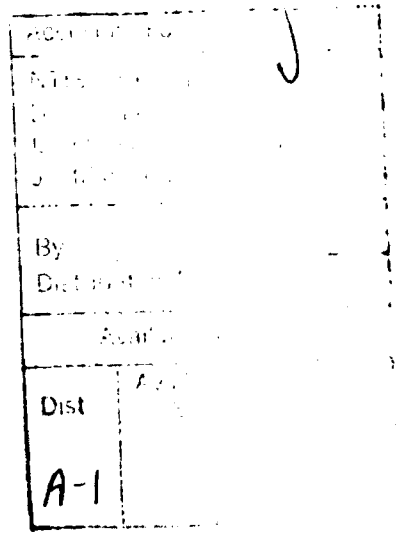

Tetrahedron Letters, Vol. 33, No. 4, pp. 441-442, 1992
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# THE ISOLATION OF A MONOMERIC CARBOXYLIC ACID OF SWINHOLIDE A FROM THE INDO-PACIFIC SPONGE, *THEONELLA SWINHOEI*

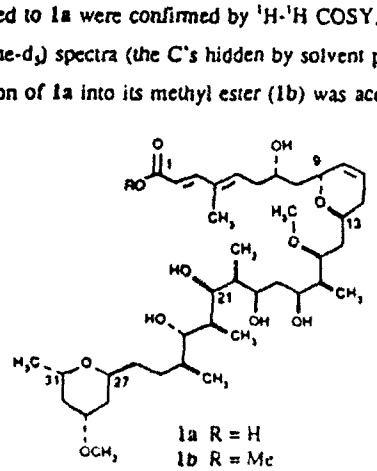
*James S. Todd,[1] K. A. Alvi, and Philip Crews**
*Department of Chemistry and Biochemistry and Institute for Marine Sciences, University of California, Santa Cruz, CA 95064*

**Abstract:** Pre-swinholide A (1a), the monomeric carboxylic acid of swinholide A (2) has been isolated from a sponge collected in Papua New Guinea. Its stereochemistry matches the absolute chirality recently established for 2.

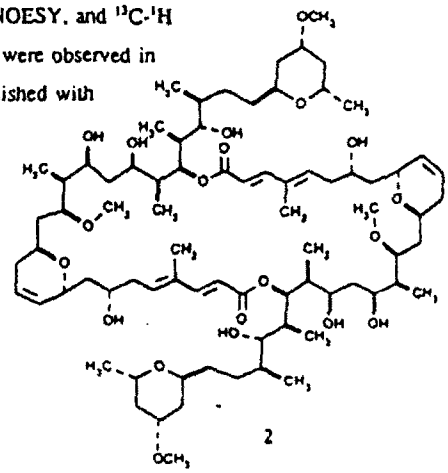
In 1988 Bergquist[2] published descriptions of 25 common shallow water sponges from Montupore Island and stated that the "history of sponge research in Papua New Guinea (PNG) is brief." Stimulated in part by this provocative statement, we began chemical investigations on soft-bodied PNG sponges. Among a small group of specimens gathered in August 1989 from Wuvulu Island offshore from Wewak was an abundant and sometimes massive sponge with one large oscula having a tan ectosome and a yellow endosome. It was identified as *Theonella swinhoei*. This species, which has been collected from both Okinawa (O) and the Red Sea (RS), is a source of: the swinholides headed by A (2) (O and RS),[3,4] a 44-membered dimeric dilactone; bistheonellides (= masakinolides) A and B, 40-membered dimeric dilactones (O);[5] theonelladins A - D, pyridine alkaloids (O);[6] the theonellins, sesquiterpene alkaloids (O);[7] and theonellapeptolides Ia - Ie, tridecapeptide lactones (O).[8]

Our collection of *T. swinhoei* (no. 89176) afforded semi-pure crude extract fractions whose $^{13}C$ NMR and LRFABMS spectra failed to reveal the presence of the simple or complex metabolites noted above. Instead, pre-swinholide A (1a), the monomeric carboxylic acid of swinholide A (2)[9] was isolated.

There were several initial clues which indicated that 1a was structurally related to 2 ($C_{78}H_{132}O_{20}$). An APT molecular formula of $C_{39}H_{62}$ was deduced for 1a by analyzing various NMR spectra. This was identical to the APT formula computed for the structure of 2. Also, the tally of eleven -C(O)H- and two -OMe residues for 1a was one half the total number of these same moieties present in 2. Of extreme significance for 1a were the molecular formula of $C_{39}H_{64}O_{11}$ established by HRFABMS ($MH^+$ 713.4855, $C_{39}H_{69}O_{11}$, $\Delta$ 1.6 mmu of calcd) and the broad IR band at 3399 $cm^{-1}$.[10] These data along with the general similarity of $^{13}C$ resonances between 1a and 2, suggested that 1a was the monomeric acid of 2. The connectivities assigned to 1a were confirmed by $^1H$-$^1H$ COSY, $^1H$ NOESY, and $^{13}C$-$^1H$ COSY NMR (pyridine-$d_5$) spectra (the C's hidden by solvent peaks were observed in MeOH-$d_4$). Conversion of 1a into its methyl ester (1b) was accomplished with



1a R = H
1b R = Me



2

# The Antifouling Activity of Natural and Synthetic Phenolic Acid Sulfate Esters

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**Key Word Index** -- Zostera marina, eelgrass, marine biofouling, p-(sulfooxy)cinnamic acid, sulfate esters, phenolic acids.

**Running Title** -- Antifouling Activity of Phenolic Acid Sulfates

Revision submitted to Phytochemistry: 12 January 1993

**Abstract** -- p-(Sulfooxy)cinnamic acid was isolated as a natural product for the first time from the seagrass Zostera marina L. (eelgrass), and was found to prevent attachment of marine bacteria and barnacles to artificial surfaces at non-toxic concentrations. Analogous synthetic sulfate esters had similar antifouling properties, while the non-sulfated phenolic acid precursors were ineffective. The antifouling properties of phenolic acid sulfates are consistent with an emerging pattern of biological activity exhibited by other sulfate esters isolated from a variety of marine organisms, and their low toxicity offers promise for the development of environmentally-benign antifouling agents to protect structures in aquatic environments.

## INTRODUCTION

Phenolic compounds are widely distr'buted in marine plants, and a number of biological functions have been attributed to them [1-5]. Examples of these natural products isolated from Zostera marina L. (eelgrass) to date include flavone sulfates [3] (the 7-sulfates of luteolin, diosmetin, apigenin, chrysoeriol, and the 7,3'-disulfate of luteolin) and non-sulfated phenolic acids [1,6] (p-coumaric, ferulic, caffeic, vanillic, gallic, protocatechuic, gentisic, 4-hydroxybenzoic, and o-pyrocatechuic acids). Previous studies with leaf extracts suggest that phenolic constituents of eelgrass may inhibit amphipod grazing,

microbial growth [7] and photosynthetic carbon uptake in epiphytic diatoms [8]. Phenolic acids may also confer resistance to the so-called "wasting disease" blamed for the catastrophic die-off of eelgrass in the North Atlantic during the 1930's [9]. Although seasonal variation in total phenolic content of eelgrass leaves does not support a strong antifouling role for these compounds in nature [10], crude methanolic extracts of eelgrass have been found to inhibit the attachment of marine bacteria, diatoms, barnacles, and polychaetes to artificial surfaces (Zimmerman, unpublished).

The goal of this study was to search for and characterize compounds present in extracts of Z. marina leaves that act to prevent the attachment of marine bacteria, epiphytic algae and invertebrates to submerged surfaces without being indiscriminate toxins. Here we report the first isolation of naturally occurring p-(sulfooxy)cinnamic acid (p-coumaric acid sulfate, 1), and compare its antifouling properties with those of non-sulfated phenolic acids and synthetic phenolic acid p-sulfate esters.

## RESULTS AND DISCUSSION

The major antifouling component isolated from the methanolic extract of eelgrass shoots was shown by a $^{13}C$ APT NMR spectrum to contain six methine and three quaterny carbons, while the $^{1}H$ NMR spectrum indicated two aromatic

proton doublets and two vynilic proton doublets. These data indicated a para-substituted trans-cinnamic acid (see EXPERIMENTAL). The unknown substituent was suspected to be a p-sulfooxy group when it was noted that the compound decomposed to p-coumaric acid (2) during HPLC purification using 0.1% aqueous TFA. A negative high resolution fast atom bombardment mass spectrum (HRFABMS) of purified natural 1 gave the predicted molecular ion. Treatment of natural 1 with diazomethane yielded the expected methyl p-(sulfooxy)cinnamate (methyl C and H at $\delta$ 50.8 and 3.72) consistent with NMR spectra and MS $M^+$ ion. Final confirmation of structure was obtained by matching the NMR data of natural 1 purified from eelgrass with those of 1 synthesized from 2 and $ClSO_3H$.

The sulfate esters of the three cinnamic acid derivatives known to occur in eelgrass (p-coumaric (2), ferulic (3), and caffeic (4) acids) were synthesized in order to determine whether the sulfate moiety was primarily responsible for the antifouling property of 1. Since the p-sulfate ester was acid-labile, synthesis was performed under basic or neutral conditions to the extent possible. Acid hydrolysis of the sulfate bond may explain previous failures to detect these compounds in eelgrass extracts by chromatographic separations performed at low pH [4]. The reaction of 4 with $ClSO_3H$ gave a mixture of the disulfate (5) and the two monosulfates (6,7). Although the monosulfates were not resolved by the TLC or HPLC conditions

employed, the presence of the two isomers was apparent from the $^{13}C$ NMR spectrum showing eighteen carbons and the expected molecular ion observed by negative HRFABMS.

Antifouling dose-effectiveness of the three synthetic phenolic acid sulfates (**1**, **5** and **8**) against a marine bacterium were statistically identical to natural **1** isolated from eelgrass (Fig. 1); the aggregate $EC_{50}$ value was approximately 10 $\mu g\ cm^{-2}$. The quantitative dose response determined by linear regression was highly significant (attached bacterial density = 76 - 0.21·log dose, $r^2 = 0.83$, $p < 0.01$). In contrast, the non-sulfated phenolic acids (**2**-**4**) showed no significant antifouling activity at concentrations 60-fold higher than the $EC_{50}$ dose for the sulfate esters (data not shown). Therefore, the presence of the sulfate ester was necessary for the antifouling function. $LD_{50}$ concentrations remain undefined because these compounds could not inhibit growth of the bacteria in liquid culture at concentrations at least three orders of magnitude higher than those required to inhibit attachment. Only the sulfate ester of ferulic acid (**8**) has been examined for its ability to inhibit attachment of barnacle (Balanus amphitrite) cyprids, and its effectiveness was similar to that observed for marine bacteria, again without toxicity (Fig. 2).

The antifouling properties of phenolic acid sulfates are consistent with an emerging pattern of biological activity attributed to sulfate esters from a variety of

marine organisms. A number of polycyclic phenolic sulfates isolated from several species of crinoids have effectively deterred predation by fish [11]. Similar studies of steroid sulfates isolated from sponges [12, 13, 14] demonstrated that the sulfate group was required for antimicrobial activity of these compounds. Although the antifouling mode-of-action of phenolic acid sulfates is currently unknown, these compounds are highly water-soluble and a solution-active mechanism is most probable. The extremely low toxicity of the phenolic acid sulfates also suggests that these compounds may provide an environmentally-acceptable means to control biofouling in aquatic environments.

## EXPERIMENTAL

Plant material. Emergent shoots of the seagrass Zostera marina L. were collected by SCUBA divers from a subtidal bed (5-7 m deep) near Del Monte Beach, in Monterey Bay, California (36° 36' 15" N, 121° 53' 10" W) in March (dry wt. 575 g) and October (dry wt. 1700 g) 1990.

Extraction and isolation. The combined dry residue from three MeOH extractions (20°) of freshly dried ground eelgrass leaves was extracted with $H_2O$ prior to being partitioned between hexanes and 10% aq. MeOH; the MeOH phase was then diluted to 40% aq. MeOH and extracted with $CH_2Cl_2$. Bioassays of these fractions indicated antifouling activity was principally localized in the $H_2O$ extract.

Lyophilization of the aq. extract gave a hygroscopic solid that was separated batchwise into three colored bands on a Sephadex LH-20 column (42 cm x 3.2 cm OD, MeOH). The compound with Antifouling activity, which was concentrated in the middle yellow band, was identified as **1** after removal of inorganic salts and other impurities by HPLC. Quantitative estimates of yield were not calculated from the March collection because preliminary experiments were performed with various fractions that resulted in acid decomposition of **1**. The October collection, however, yielded 66 mg of **1** from 1700 g dry biomass.

Chromatography. Final purification of all phenolic acid sulfates (natural and synthetic) was carried out by HPLC [Regis ODS irregular column, 25 cm x 10 mm ID; RI detector; 90% aq. MeOH solvent (100% $H_2O$ for **5-7**); 1000 psi]. TLC $R_f$ values [silica gel; BuOH-HOAc-$H_2O$ (4:1:1); UV detection] : **1** (0.63); **2** (0.83); **3** (0.75); **4** (0.80); **5** (0.29); **6** and **7** (0.61); (**8**) (0.58).

Antifouling Bioassays. Frosted ends (4.5 $cm^2$) of glass microscope slides were treated with candidate fractions or purified compounds dissolved in MeOH and challenged against a clone of *Acinetobacter* sp., a fouling marine bacterium isolated from the surface of eelgrass leaves. Control slides were treated with MeOH solvent only. Slides were placed into 50 mL screw-cap plastic tubes containing 30 mL of sterile, 0.2 μm-filtered seawater (FSW) and an inoculum of bacteria from a log-phase liquid culture (final bacterial

concentration was ca. $10^6$ cells $mL^{-1}$). Tubes were capped and placed horizontally on a rotary shaker with the treated surface facing down. No attempt was made to stabilize or otherwise prevent the dissolution of material from the treated surfaces. Slides were removed at 20 min intervals, stained with Hoechst (#2287, Sigma Co.) and cell densities in the frosted regions were enumerated with the aid of epifluorescence microscopy (1000x). Attached bacterial densities on treated slides were normalized to control slides at each time point. Mean density (relative to MeOH control) was calculated for each concentration from all data in the 4 h time series.

Barnacle attachment assays were performed in plastic petri dishes treated with 8 and control solvent (MeOH). Dishes were then filled with FSW and competent cyprid of Balanus amphitrite were added. As with the bacterial assays, no attempt was made to stabilize or otherwise prevent the dissolution of material from the treated surfaces. After 24 h, the number of attached cyprids in each dish was determined, and normalized to the number added initially. Ten replicate dishes were analyzed for each concentration.

Synthesis of 1. $ClSO_3H$ (0.2 ml) was added dropwise to 2 (200 mg) in pyridine (0.5 ml) with stirring at 20°. Ice $H_2O$ was added, and the acidic aq. mixture extracted with $Et_2O$, basified, extracted with $Et_2O$, and $H_2O$ removed under vacuum. Residue was triturated with $H_2O$, neutralized, dried under

vacuum, and triturated with MeOH. MeOH soluble residue purified by HPLC gave 187 mg 1 (63%). Similar yields obtained for 5 - 8.

Spectral data. All mass spectra were negative HRFAB. Matrix: thioglycerol/glycerol (only glycerol for 5-7). NMR * assignments interchangeable.

Natural 1: $[M-1]^-$ 242.9963, $C_9H_7O_6S$, $\triangle$ 0.0 mmu. APT and $^{13}C$ NMR (62.5 MHz, $CD_3OD$-$D_2O$): $\delta$ 176.5 (s, C-1), 153.5 (s, C-7), 140.8 (d, C-3), 134.3 (s, C-4), 130.2 (2C, d, *C-5), 125.8 (d, C-2), 122.9 (2C, d, *C-6). $^1H$ NMR (300 MHz, $CD_3OD$-$D_2O$): $\delta$ 7.59 (2H, d, $J$=8.7 Hz, *H-5), 7.34 (1H, d, $J$=16.2 Hz, H-3), 7.27 (2H, d, $J$=8.4 Hz, *H-6), 6.44 (1H, d, $J$=16.2 Hz, H-2).

Methyl ester of natural 1: $[M-1]^-$ 257.0118, $C_{10}H_9O_6S$, $\triangle$ 0.2 mmu of calculated. $^{13}C$ NMR (75 MHz, $CD_3OD$): $\delta$ 167.8, 154.5, 144.3, 130.7, 128.9 (2C), 121.2 (2C), 116.5, 50.8. $^1H$ NMR (300 MHz, $CD_3OD$): $\delta$ 7.65 (d, $J$=15.9 Hz), 7.57 (d, $J$=8.7 Hz), 7.31 (d, $J$=8.7 Hz), 6.45 (d, $J$=15.9 Hz), 3.72 (s).

Synthetic 1: $[M-1]^-$ 242.9958, $C_9H_7O_6S$, $\triangle$ 0.5 mmu of calculated. NMR spectra same as natural 1.

5: $[M-2H+Na]^-$ 360.9285, $C_9H_6NaO_{10}S_2$, $\triangle$ 1.5 mmu of calculated. $^{13}C$ NMR (62.5 MHz, $D_2O$): $\delta$ 176.5, 144.9, 144.3, 140.3, 135.3, 127.4, 124.4, 123.3, 123.1. $^1H$ NMR (250 MHz, $D_2O$): $\delta$ 7.81 (br s), 7.62 (br s), 7.45 (d, $J$=16.0 Hz), 6.61 (d, $J$=16.0 Hz).

**6** and **7** mixture: $[M-1]^-$ 258.9888, $C_9H_7O_7S$, $\triangle$ 2.4 mmu of calculated. $^{13}C$ NMR (62.5 MHz, $D_2O$): $\delta$ 177.0, 176.7, 151.0, 149.4, 141.2, 140.9, 140.1, 135.4, 128.9, 128.1, 127.2, 125.7, 124.1, 123.5, 123.0, 121.4, 118.8, 117.0. $^1H$ NMR (250 MHz, $D_2O$): $\delta$ 7.74 (s), 7.59 (d, $J$=2.0 Hz), 7.56 (s), 7.42-7.35 (m), 7.30 (d, $J$=3.2 Hz), 7.22 (d, $J$=1.9 Hz), 7.18 (d, $J$=2.0 Hz), 7.15 (d, $J$=2.0 Hz), 7.03 (d, $J$=8.4 Hz), 6.53 (d, $J$=18.3 Hz), 6.41 (d, $J$=14.8 Hz).

**8**: $[M-1]^-$ 273.0083, $C_{10}H_9O_7S$, $\triangle$ 1.4 mmu of calculated. $^{13}C$ NMR (62.5 MHz, $CD_3OD$): $\delta$ 175.6, 153.1, 143.5, 140.2, 134.8, 126.3, 123.5, 121.2, 112.5, 56.6. $^1H$ NMR (250 MHz, $CD_3OD$): $\delta$ 7.40 (d, $J$=8.3 Hz), 7.30 (d, $J$=15.9 Hz), 7.14 (d, $J$=1.9 Hz), 7.02 (dd, $J$=8.4, 1.9 Hz), 6.40 (d, $J$=15.9 Hz), 3.83 (s).

Acknowledgements. We thank G. Suba and G. Kraemer for assistance with field collections, S. Fain and C. Dea for assistance with the biofouling assays, A. Claire for performing the barnacle attachment assays, and J. Loo for HRFABMS. This research was supported by contracts from the Office of Naval Research (N00014-88-K-0445) and Office of Naval Technology (N00014-90-J-1344 & N00014-91-J-1815).

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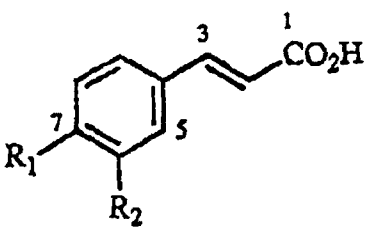
## FIGURE LEGENDS

Figure 1. a) Antifouling dose-response of (1) natural p-(sulfooxy)cinnamic acid (●), synthetic (1) p-(sulfooxy)cinnamic acid (○), (8) p-(sulfooxy)ferulic acid (△), and (5) 3,4 (disulfooxy)caffeic acid (□) relative to control slides, against Acinetobacter sp. Lines indicate linear regression of log-transformed data and 99% confidence limits.

Figure 2. Antifouling dose-response curve for (8) synthetic p-(sulfooxy)ferulic acid (●) against barnacle (Balanus amphitrite) larvae in laboratory assays, and solvent controls (○).






| | $R_1$ | $R_2$ |
|---|---|---|
| 1 | $OSO_3H$ | H |
| 2 | OH | H |
| 3 | OH | OMe |
| 4 | OH | OH |
| 5 | $OSO_3H$ | $OSO_3H$ |
| 6 | $OSO_3H$ | OH |
| 7 | OH | $OSO_3H$ |
| 8 | $OSO_3H$ | OMe |

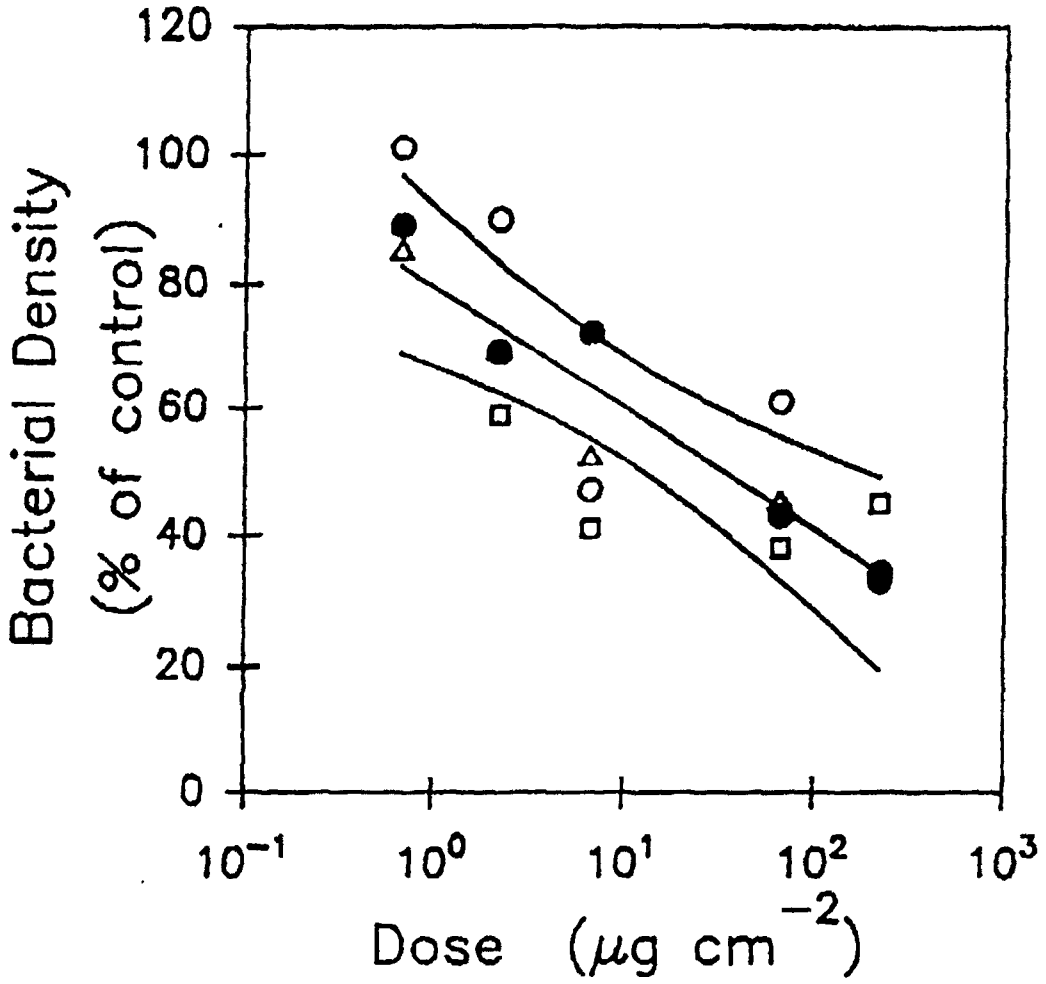



Figure 1; MS#977, Todd, Zimmerman, Crews & Alberte

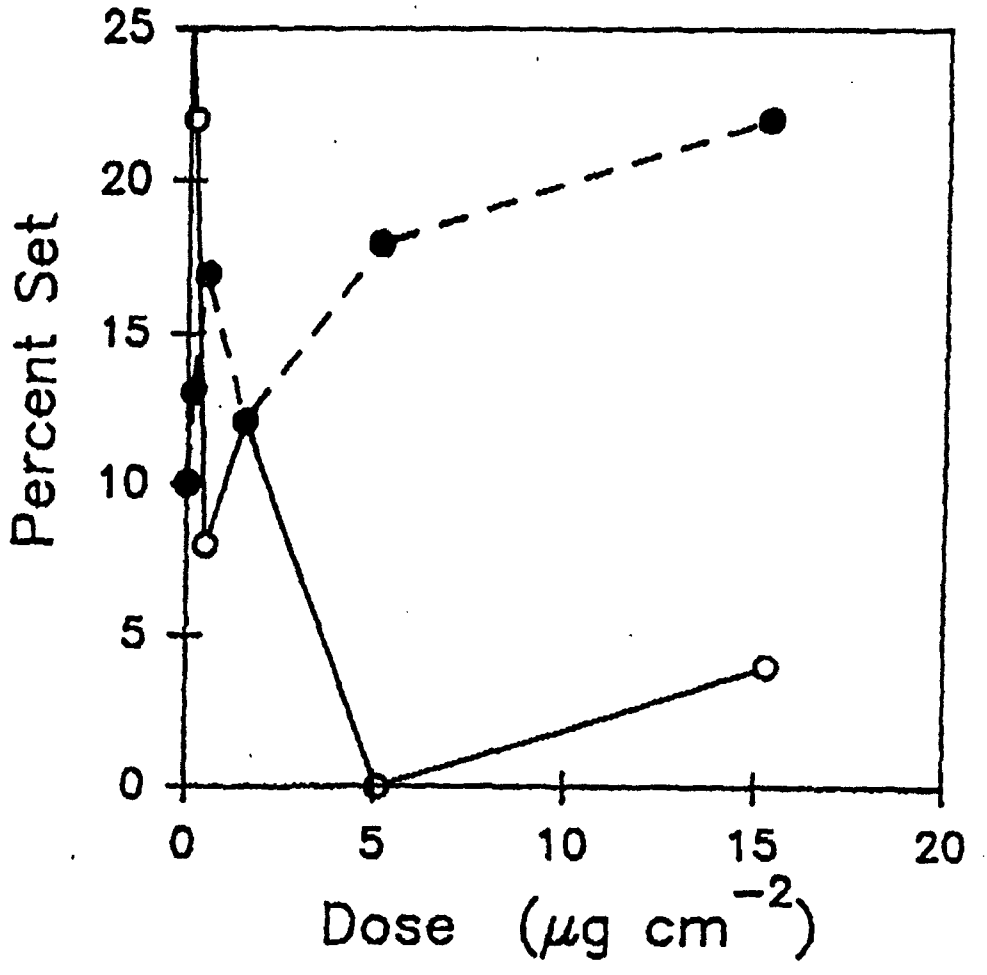



Figure 2, Ms #977, Todd, Zimmerman, Crews & Alberte